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Review

The virally encoded killer proteins from *Ustilago maydis*

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ABSTRACT

Several strains of *Ustilago maydis*, a causal agent of corn smut disease, exhibit a 'killer' phenotype that is due to persistent infection by double-stranded RNA Totiviruses. These viruses produce potent killer proteins that are secreted by the host. This is a rare example of virus/host symbiosis in that these viruses are dependent upon host survival and, to that end, produce antifungal proteins that kill competing, uninfected strains of *U. maydis*. Two of the best-studied examples of this killer phenomenon are *U. maydis* strains P4 and P6 that secrete killer proteins KP4 and KP6, respectively. The mature form of KP4 is comprised of 105 residues while KP6 consists of two subunits, *a* and *b* chains, 76 and 82 residues in length, respectively. KP6 is not homologous to any known protein, and only recently has KP4 been shown to have possible homologs in pathogenic fungi. While very little is known as to the mode of action of KP6, we have shown that KP4 blocks L-type Ca^{2+} channels in fungi and animal cells in a reversible and cytostatic manner. In contrast, preliminary results suggest that KP6 acts via a completely different mechanism and is a potent cytolytic antifungal protein. When KP4 is expressed in maize, the resulting transgenic lines are nearly immune to *U. maydis* infection. Therefore, a greater understanding of the modes of action of these potent antifungal proteins could lead to development of broad-spectrum antifungal agents.

1. Introduction

The life cycle of the fungus *Ustilago maydis*, corn smut, has three phases: diploid, haploid, and dikaryon. While dikaryon formation and development is restricted to host (maize) tissue, the diploid and haploid mycelia grow rapidly in a yeast-like manner in laboratory media. A transient dikaryon (or heterokaryon) may be formed between compatible strains on laboratory medium. *U. maydis* is heterothallic. Sexual reproduction between two haploid strains depends on two unlinked genes. The *a* gene has two alleles and the *b* gene has multiple alleles (Rowell and Devay, 1954; Schulz *et al.*, 1990). Cells with opposite *a* alleles will fuse under appropriate conditions to form a heterokaryon which will infect the host and allow the completion of the life cycle only if the *b* alleles are also different (Puhalla, 1968). The *a* gene can, therefore, be regarded as the mating type locus, homologous to the simple two-allele mating systems of most yeast, whereas

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the *b* gene controls the development of the dikaryon in infected host cells, which is the only place that sexual development can proceed.

Killer toxins are produced by several genera of yeast (Young, 1987) and filamentous fungi (de Sa *et al.*, 2009, 2010). A small proportion of *U. maydis* strains produce killer toxins, to which they are resistant, but sensitive strains are the vast majority in wild populations. There are three killer types: P1, P4 and P6, that secrete KP1, KP4 and KP6 toxins, respectively. Correspondingly, there are three groups of resistant cells, in which resistance is determined by three independent recessive nuclear genes: p1r, p4r and p6r (Koltin and Day, 1976; Puhalla, 1968). The locations and sequences of the resistance genes are unknown. For example, cells cannot produce KP6 unless they have the p6r gene (Finkler *et al.*, 1992) and the same is true of KP4. Since these putative receptors are different in each case, there is no cross-resistance to the toxins. This is supported by the fact that there are no single resistance alleles that confer simultaneous resistance to all three toxins. Interestingly, all three nuclear genes that confer resistance to these antifungal proteins are recessive to their sensitive alleles (Koltin and Day, 1976). Sexual crosses and heterokaryons between different killer strains demonstrated that restrictions prevent inclusion of two killer specificities in the same cell.

What makes these antifungal proteins so unusual compared to plant and animal antifungal proteins is that the *U. maydis* killer toxins are produced only by strains with dsRNA viruses (UmV) in the cell cytoplasm (Koltin and Day, 1976). Most fungal dsRNA viruses do not confer resistance to their host cells or any characteristic phenotype. They are persistent, segmented dsRNA viruses whose segments are separately encapsidated and whose only means of transmission is through mitosis or meiosis. Of these dsRNA viruses the *S. cerevisiae* virus (ScV) and the *U. maydis* virus (UmV) are known to encode killer toxins (Koltin and Day, 1976; Wickner, 1989).

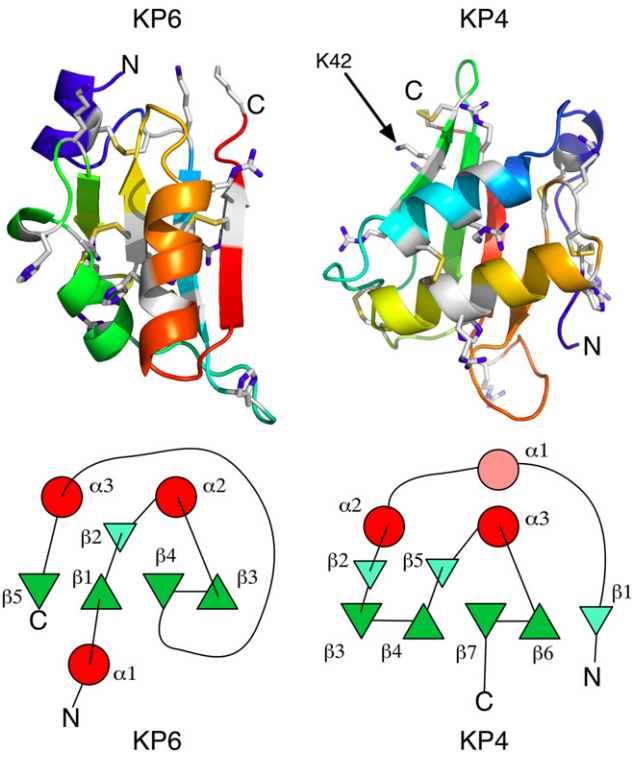

Fig. 1 – Atomic structures of the α subunit of KP6 (left) and KP4 (right). The top figures are ribbon drawings of the two atomic structures colored from blue to red as the polypeptide chain proceeds from the N to the C termini. Also shown are the side chains of the basic residues and the disulfide bonds found in the proteins. Residue K42 is indicated in KP4 and is located at the base of the C-terminal protrusion that is similar to the active site found on scorpion toxin. The K42Q mutation abrogates KP4 activity. At the bottom are protein fold schematics comparing the general secondary structure patterns of the two proteins.

2. Atomic structures of KP4 and KP6

The structure of KP4 was first determined (Gu *et al.*, 1995) and shortly followed by that of the α subunit of KP6 (Li *et al.*, 1999) (Fig 1). The KP4 toxin is different from the other *U. maydis* toxins and is the one that much is now known about. It is a single polypeptide of 105 amino acids (Park *et al.*, 1994) and, unlike KP1 and KP6, is not processed by Kex2p (Park *et al.*, 1994). There is no sequence similarity between KP4, KP6, and other known toxins (Ganesa *et al.*, 1991; Park *et al.*, 1994). While most of the yeast toxins are acidic (Bussey, 1972) and the KP6 and KP1 toxins have neutral pI's (Levine *et al.*, 1979), KP4 is extremely basic with a pI > 9.0. At the time, KP4 was found to have a unique compact α/β sandwich structure held together by five disulfide bonds (Gu *et al.*, 1995). KP4 belongs to the α/β-sandwich family of proteins and has a single split βαβ motif with a total of seven β-strands (131–137) and three α-helices (α1–α3). One of the most unusual features of the protein is that the two major helices, α2 and α3, have a left-handed βαβ crossover conformation. The possible biological function of these unusual arrangements are unclear, it may be to create a 'cup-like' surface on the opposite side of the β-sheet that might be important for protein–protein interactions.

The KP6 α-subunit also does not share any sequence homology with KP4 and its structure only bears modest similarity to KP4 (Li *et al.*, 1999) (Fig 1). KP6 α-subunit forms a single domain structure that has an overall ellipsoid shape and also belongs to the α/β-sandwich family. The tertiary structure consists of a four-stranded antiparallel β-sheet, a pair of antiparallel α-helices, a short strand along one edge of the sheet, and a short N-terminal helix. Although the fold is reminiscent of toxins of similar size, the topology of KP6α is distinctly different in that the α/β-sandwich motif has two right-handed βαβ split crossovers. Crystallographic symmetry forms hexamers of KP6α with a central pore lined by the hydrophobic N-terminal helices. Li *et al.* (1999) suggested that this central pore could play an important role in the mechanism of the killing action of the toxin. However, the location and role of the β-subunit in this assembly were unknown.

3. Effects of KP4 on *U. maydis* cells

The dogma had been that KP4 affects *U. maydis* cells by forming pores in the cell membrane like colicin and some of the small anti-microbial peptides found in animals (Aerts *et al.*, 2008) and yeast (Walker *et al.*, 1995). However, from its structure this seemed unlikely. KP4 is only 105 residues in length but contains five disulfide bonds and is highly charged (Gu *et al.*, 1995). From this, it seemed unlikely that it would form channels on its own or, because of the disulfide bonds, be able to undergo the kinds of conformational changes required to form pores in the membrane. Because of a few similarities to the scorpion toxins, we proposed that KP4 might affect some other channel at the membrane surface (Gu *et al.*, 1995). To test for this, cell growth was measured in the presence of KP4 with increasing concentrations of K^+, Na^+, Mg^{2+}, and Ca^{2+} (Fig 2). Indeed, Ca^{2+} was very effective at abrogating KP4 inhibition, but, with the exception of a slight effect by K^+, none of the other metals had any effect even at very high concentrations (Gage *et al.*, 2001; Gu *et al.*, 1995). This suggested that KP4 might be blocking Ca^{2+} channels. If true, then this further suggests that the block is via reversible protein–protein interactions with the Ca^{2+} channels. To test this hypothesis, sensitive P2 cells were treated for more than 24 h with high concentrations of KP4 and then extensively washed with minimal media. When treated in this way, the cells did recover, but with about a 6 h delay. We then added Ca^{2+} to the wash and found that this addition made the washing as effective as if the cells had never been treated with KP4 at all (Gage *et al.*, 2001).

These results strongly suggest that KP4 reversibly acts on membrane proteins that are likely to be Ca^{2+} channels. More specifically, we also demonstrated that KP4 inhibited uptake of $^{45}Ca^{2+}$ in *U. maydis cells* (Gage *et al.*, 2001). We followed up these assays with animal cells where electrophysiology is more tractable without the cell wall and the various types of Ca^{2+} channels are well characterized.

4. KP4 blocks L-type voltage-gated Ca^{2+} channels

Perhaps our most surprising and convincing evidence that KP4 acts on Ca^{2+} channels was that KP4 specifically blocks voltage-gated animal channels (Gage *et al.*, 2002). KP4 blocked $Ca_v1.2$,

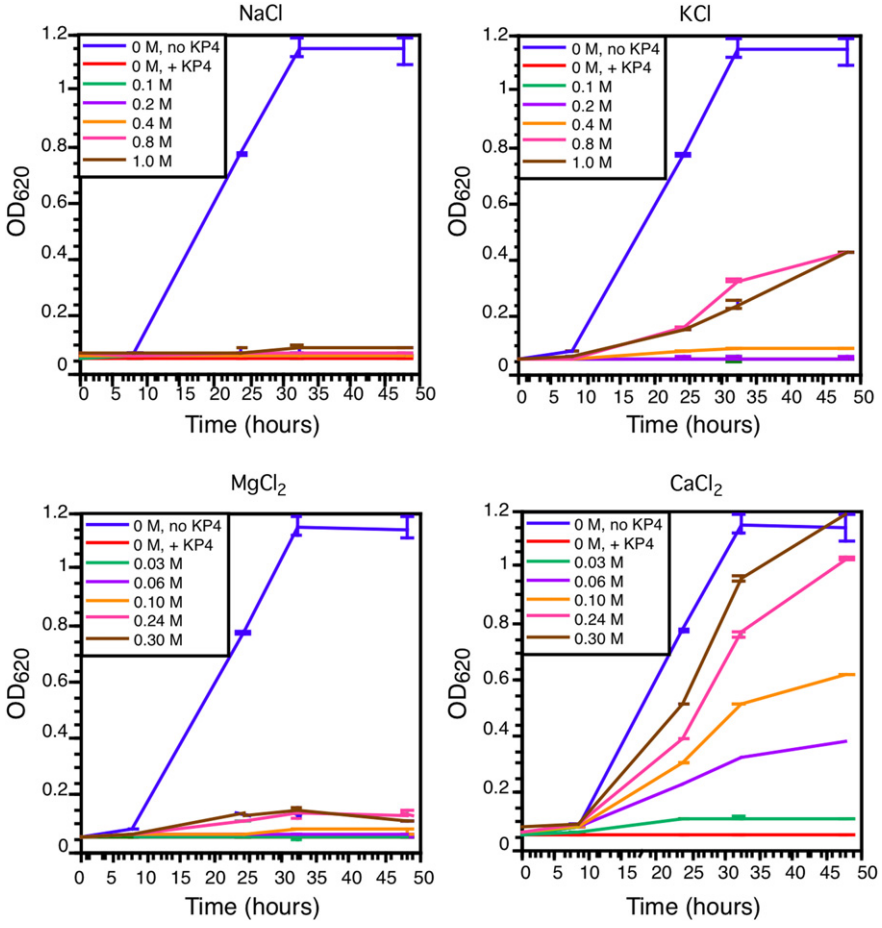

Fig. 2 – Effects of metals on KP4 killing activity. Shown here are the growth curves for the KP4-sensitive strain, P2, in the presence and absence of KP4 and at varying concentrations of metal in the media. All concentrations represent the calculated ionic strength of the added metals to demonstrate that the abrogation of KP4 killing is not merely a charge shielding effect. Only calcium is able to strongly abrogate the effects of KP4.

but had no effect on $Ca_v2.1$ or $Ca_v2.3$ channels. KP4 specifically blocks L-type Ca^{2+} channels with weak voltage-dependence to the block. In a manner akin to the calciseptine peptide block of $Ca_v1.2$, KP4 does not affect the voltage-dependence of activation but slightly shifts the voltage-dependence of inactivation of the channel (Teramoto *et al.*, 1996). KP4 activity against both mammalian and fungal cells was blocked by chemical modification of the single lysine residue – akin to what was found with scorpion toxin (Sampieri and Habersetzer-Rochat, 1978). Further, as was observed in fungi, the inhibition of the animal channel was abrogated by the addition of Ca^{2+} (Fig 3). Interestingly, at the time of the publication of this paper, we suggested that Cch1 was the likely target for KP4 since Cch1 showed homology to the mammalian voltage-gated L-type Ca^{2+} channels (Gage *et al.*, 2002).

Over the past decade, it has been shown that nearly all fungi contain a gene that is homologous to the catalytic α-subunits of voltage-gated Ca^{2+} channels in animals (Fischer *et al.*, 1997; Paidhungat and Garrett, 1997). Typically, these animal channels are voltage-gated and open in response to changes in the transmembrane electrical potentials. The fungal homologs, such as Cch1 in *S. cerevisiae*, contain half as many cationic residues in their voltage-sensing S4 segments as mammalian homologs, suggesting gating and Ca^{2+} influx in fungi may respond to an unusual voltage range or other kinds of stimuli. In addition, *S. cerevisiae* and other yeasts require Mid1, a protein with sequence homologs present only in fungi and a secondary structural subunit that is similar to the $\alpha 2\delta$ -subunits of animal voltage-gated Ca^{2+} channels.

Recently, it was shown that the Cch1 channel from the model human fungal pathogen, *Cryptococcus neoformans*, is activated by the depletion of intracellular Ca^{2+} stores (Hong *et al.*, 2010). Using electrophysiological analysis it was shown that agents that enable endoplasmic reticulum Ca^{2+} store depletion promote the development of whole cell inward Ca^{2+} currents through Cch1 that are effectively blocked by La^{3+} and dependent on the presence of Mid1. This study demonstrated that Cch1 functions as a store-operated Ca^{2+}-selective channel that is gated by intracellular Ca^{2+} depletion. The inability of cryptococcal cells lacking the Cch1–Mid1 channel to survive ER stress suggests that Cch1 and its co-regulator, Mid1, are critical players in the restoration of Ca^{2+} homeostasis. This is consistent with the idea that the Cch1 channel is a high affinity Ca^{2+} transporter but is not voltage-gated. It may be that Cch1 is a target for KP4 or plays some role in its effects.

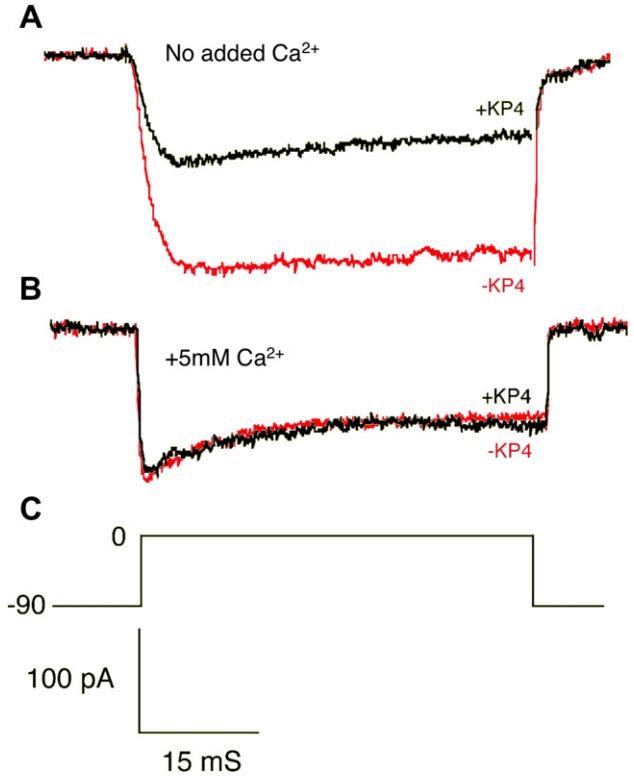

Fig. 3 – Effects of KP4 on mammalian cells. (A): a representative calcium current traces from rodent pituitary-derived GH3 cells, predominantly expressing L-type calcium channels, with 10 mM Ba^{2+} in the extracellular bath solution. (B): representative traces from GH3 cells where the Ba^{2+} is replaced with 5 mM Ca^{2+} in the extracellular bath solution. (C): The step function of voltage applied during the whole cell electrophysiology experiments and the very bottom shows the scale bars for current and time. These effects of KP4 effects, as with its antifungal activity, are abrogated by the presence of Ca^{2+} in the bath solution.

5. Effect of KP4 on plants

Since KP4 affects not only fungal but mammalian calcium homeostasis, we wanted to see if it might also block plant calcium channels. To this end, a non-intrusive and real-time method was chosen to monitor the effects of these antifungal proteins on tip-growing root hair cells. The Nielsen lab had previously characterized a transgenic line of *A. thaliana* expressing an enhanced yellow fluorescent protein/Rab GTPase fusion protein; EYFP-RabA4b (Preuss *et al.*, 2004). RabA4b is involved in a vesicular transport and is localized to the tips of growing root hairs. Tip-localized EYFP-RabA4b disappears in mature root hair cells that have stopped expanding. As in fungal hyphae, the root hair tip growth is associated with an apex-high cytosolic free calcium gradient generated by a local influx of calcium at the tip (Felle and Hepler, 1997; Schiefelbein *et al.*, 1992). This calcium gradient was shown to be crucial for RabA4b localization since disruption of the calcium gradient causes a concomitant abrogation of the RabA4b gradient (Preuss *et al.*, 2006, 2004). Therefore, the effects of these antifungal proteins on the localization of the fluorescent EYFP-RabA4b fusion protein to the apical tip of the root hair offers an indirect and non-invasive means to monitor the effects of defensins and KP4 on the calcium gradient in growing root hair cells.

As shown in Fig 4, the addition of KP4 to growing root hairs causes rapid dissipation of the EYFP-RabA4b gradient at the apical tip and a concomitant cessation of root hair elongation. The EYFP-RabA4b gradient is dissipated within 2 min and the root hair extension stops shortly thereafter. In all cases the EYFP-RabA4b gradient can be reestablished and root hair extension resumed after KP4 was removed. KP4 completely

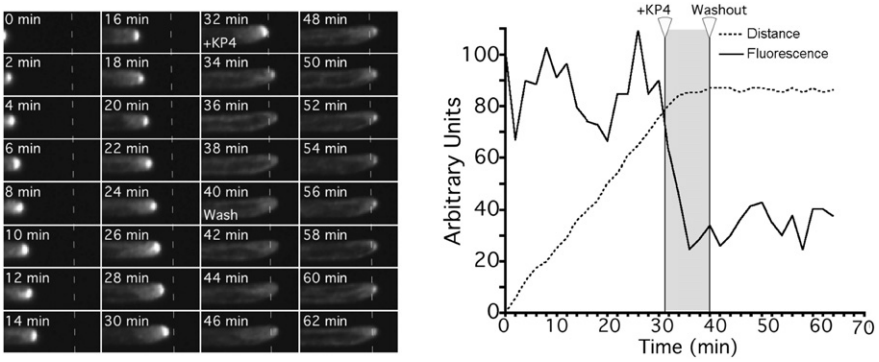

Fig. 4 – Effects of KP4 on RabA4b localization and root hair growth. Shown here are the effects of KP4 on both RabA4b polarization and extension of *A. thaliana* root hair. The aligned, raw images are shown on the left side of each panel and a graph summarizing the growth and fluorescence of the apical tip is shown on the right. For this experiment, RabA4b localization at the root hair tip was monitored for ~30 min and then 0.5 μM KP4 and then washed out after ~10 min. The position of the root hair tip at the time the antifungal protein was added is noted by the dashed white line on the left side of the panels and growth of the root hair is represented by the black dashed line in the graph on the right side of the panels. The average intensity of the pixels at the apical tip, representing the polarization of EYFP-RabA4b, is shown as a solid line on the graph. Not shown, the experiment was continued for 2 h to demonstrate that growth and RabA4b polarization eventually recovered after wash out.

depolarizes the EYFP-RabA4b tip-localization in less than 3 min and block root hair extension shortly thereafter. As a control, 5.0 μM BSA was added to the root hairs and did not have any effect on the EYFP-RabA4b gradient or root hair extension. The speed of this KP4 effect suggests that its target is at the outer membrane where it can rapidly bind and cause its effects. Again, this is consistent with KP4 blocking a calcium import channel. Therefore, all of these results strongly point to KP4 affecting a calcium channel. It should be noted, however, that there are not any CCH-1 or L-type calcium channel homologs in plants. Therefore the interactions between KP4 and plant root hairs may be via a localized and highly conserved portion of a calcium channel.

6. Effects of KP6 on *U. maydis* cells

There is very little known about how KP6 affects *U. maydis* cells. Previous studies have eliminated possible chitinase or protease activity (Steinlauf *et al.*, 1988), not to mention that the structure of the KP6α subunit shares no homology to any of these enzymes. It is also apparent that KP6 acts in a manner wholly different to that of KP4. While 100 mM $CaCl_2$ completely blocks KP4 inhibition and as little as 20 mM $CaCl_2$ is sufficient to cause significant abrogation (Gage *et al.*, 2001), none of the metals had any significant effect on the KP6 killing of *U. maydis* (unpublished data). The only published data on the mode of action of KP6 suggested that it might be having a strong effect on the membrane integrity or osmotic regulation (Steinlauf *et al.*, 1988). In this study, when the cells were treated with KP6 for several hours, a significant decrease in the cytoplasmic volume was observed by light microscopy. When analyzed in the scanning electron microscopy, it was noted that the first effect of KP6 was that the normally smooth cell wall became 'wavy' and with continued treatment, the cells appeared to burst. Interestingly, previous publications, and in our hands as well, the optical density of the cells drops initially in the first few hours of KP6 treatment (Peery *et al.*, 1987). Again, this is entirely consistent with KP6-induced cell lysis decreasing the light scattering of the cells. Therefore, all of the evidence clearly shows that the mode of action of KP6 is completely different to that of KP4.

What is not at all clear is how KP6 is acting on the target cells. In the previously published studies (Steinlauf *et al.*, 1988), spheroplasts were made and then treated with KP6. These studies revealed that the spheroplasts were insensitive to KP6 but when the cell wall was given time to regenerate, then the cells were once again sensitive to KP6. From this observation, it was inferred that some sort of recognition site was on the cell wall that then targeted KP6 to the appropriate cellular target. One possible problem with these results is that the spheroplasts were generated using Novozyme 234 which is no longer commercially available. Novozyme contained α-1,3-glucan cyanohydrolase to degrade the cell wall but also had low quantities of cellulase, xylanase, β-1,3-glucan glucanohydrolase, proteases, and DNase. Therefore, it is possible that there was still significant residual protease activity that digested a cell membrane protein receptor for KP6 and this could be why spheroplast were resistance to KP6. Ongoing structure/function studies are working toward better understanding the mode of action of this potent cytolytic antifungal protein.

7. Transgenic maize plants expressing KP4 exhibit robust resistance to *U. maydis*

Maize smut is a global disease responsible for extensive agricultural losses. Control of this disease using traditional breeding has met with limited success because natural resistance to *U. maydis* is organ-specific and is quantitatively inherited. In spite of the apparent inhibitory effect of adding

KP4 exogenously to *Arabidopsis* root hairs, we created several lines of transgenic maize to see whether constitutive expression of KP4 would confer resistance to *U. maydis* infection (Allen *et al.*, 2011) (Fig 5). To target KP4 to the extracellular space of transgenic maize, a monocot codon-optimized KP4 gene containing the secretory signal peptide sequence of a plant defensin MsDef1 was placed under the control of a constitutive maize *Ubi1* promoter. This gene was introduced into maize inbred line H99 and then backcrossed into B73. Transgenic maize plants expressed high levels of extracellular KP4 with no apparent negative impact on plant development. Indeed, small samples of the transgenic leaf tissue contained a sufficient amount of KP4 to produce clearing zones in the when applied to wells cut into agar containing a suspension of toxin-sensitive P2 *U. maydis* cells. The fact that we did not see any phenotypic effect of KP4 on root development could be due to less sensitivity of maize roots to KP4, possible adaptation of the plants to KP4 expression, or that the local concentration of KP4 around the roots is significantly lower than in the experiments described above.

Since transgenic maize lines secreted bioactive KP4 protein, we next determined the ability of this protein to protect transgenic lines from the corn smut disease. Ten transgenic maize lines were tested against *U. maydis* infection in the greenhouse. In general all transgenic maize lines expressing KP4 developed normally when compared to the nontransgenic control line BC_1F_4. Seven-day-old seedlings were inoculated with a mixture of the wild-type *U. maydis* KP4-sensitive strains 1/2 and 2/9 (Gold *et al.*, 1997). These strains are commonly used wild-type laboratory strains of *U. maydis* (Brefort *et al.*, 2009). Both strains, 1/2 (same as strain 521) (Kronstad and Leong, 1989) and the near-isogenic strain 2/9 (Gold *et al.*, 1997) generated galls in nontransgenic maize plants.

Disease symptoms were observed and scored at 10 days post-inoculation (dpi). To determine whether plants resisted or simply delayed *U. maydis* infection, transgenic plants were scored for disease symptoms at 21 dpi. Disease symptoms were absent at 21 dpi in several transgenic maize lines, while the wild-type line BC_1F_4 exhibited plant death (Fig 5).

Table 1 – Disease symptoms caused by *U. maydis* infection on KP4 transgenic maize.

Maize events	# Plants	Disease score						Disease index
	Total	0	1	2	3	4	5	
947*	53	49	4	0	0	0	0	0.08[b]
1040*	59	54	5	0	0	0	0	0.08[b]
972**	40	34	6	0	0	0	0	0.15[b]
885**	40	32	8	0	0	0	0	0.20[b]
851*	60	47	12	1	0	0	0	0.23[b]
810**	40	13	16	7	4	0	0	1.05[ab]
923**	40	15	12	8	5	0	0	1.08[ab]
746*	60	18	24	12	6	0	0	1.10[ab]
759**	58	9	20	19	7	3	0	1.57[ab]
826	40	2	5	3	12	17	1	3.00[a]
H99/B73 (wt)	60	2	11	10	22	13	2	2.65[a]
Non-infected 947	18	15	3	0	0	0	0	0.17[b]
Non-infected wt	20	18	2	0	0	0	0	0.10[b]

Results from three pathogenicity experiments. The disease ratings are as follows: 0 = no symptoms; 1 = anthocyanin and/or chlorosis; 2 = small leaf galls; 3 = small stem galls; 4 = basal stem galls; 5 = plant death. H99/B73 (BC_1F_4) is the wild-type (wt) maize line from which KP4 transgenic plants were generated. Symptoms were scored 10 dpi. Values with different superscript alphabets in the disease index column are significantly different ($p \leq 0.05$) in Duncan's Multiple Range Test. The numbers of plants used differed because not all seeds germinated or (in the case of uninfected plants) so many replicates were not needed.
*Lines that were homozygous at the time of testing.
**Lines that were hemizygous at the time of testing.

Five KP4 transgenic lines, 851, 947, 1040, 885, and 972 showed strong resistance to *U. maydis* infection (Table 1). Four KP4 transgenic lines, 746, 759, 810, and 923 showed moderate resistance to this fungus. One KP4 transgenic line, 826, did not confer resistance to *U. maydis* infection. Transgenic maize plants were then transplanted into large pots and compared with the wild-type plants for any developmental defects. Three-month-old transgenic maize plants developed normally when compared with wild-type plants. Secondary inoculation with 1/2 and 2/9 strains directly into maize ears was done upon the appearance of silks. Two weeks later, plant

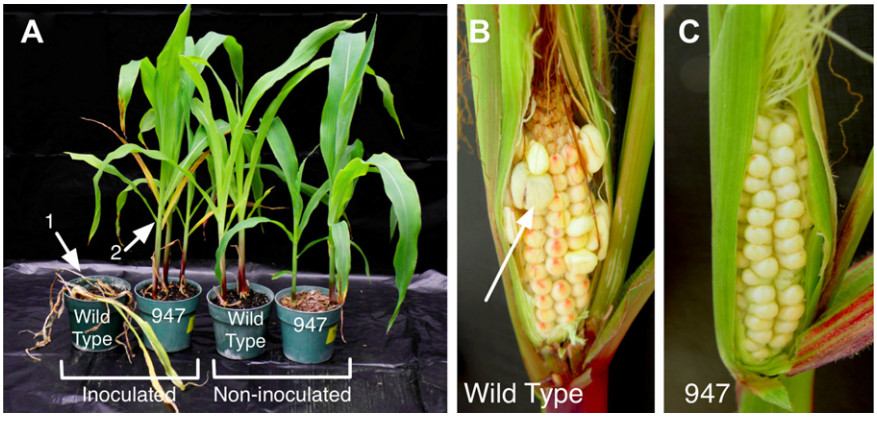

Fig. 5 – KP4 transgenic plants are highly resistant to *U. maydis* infection. (A) Wild-type (BC_1F_4) maize seedlings 21 days post stem inoculation results in death (white arrow 1) while the KP4 transgenic plants remain symptomless (line 947; white arrow 2). With wild-type maize, galls appear 14 days post inoculation in the wild-type (B; arrow), while the KP4 transgenic maize remain free of disease for the duration of the experiment (C).

tumors or galls were observed in the ears of the wild-type line (BC_1F_4) and transgenic line 826, while the ears of the KP4 transgenic lines 851, 746, 759, 947, 1040, 810, 885, 923 and 972 remained healthy and disease-free. Indeed, challenges of highest expressing lines of maize yielded the same disease score as the uninfected wild-type plants (Table 1). These results suggest that a high level of organ independent, broad-spectrum, and durable maize smut resistance might be afforded by transgenic co-expression of KP4 and other Totivirus antifungal proteins, KP1 and KP6.

8. Conclusions

These results demonstrate that this family of naturally expressed antifungal proteins holds promise in identifying new targets for antifungal agents and a novel means to protect crops against fungal infections. From the basic science standpoint, it is fascinating to consider where these viruses acquired these antifungal proteins and how the host evolved both resistance to their effects as well as the process of accommodating the persistent *Totivirus* infection. Recently, it was found that several KP4-like proteins are part of the predicted secretome of *Fusarium graminearum* (Brown *et al.*, 2012). This suggests that KP4-like proteins are far more ubiquitous than previously thought. Further, it also suggests that the ancestral precursor to KP4 may have come from a completely different fungal species and this implies that these dsRNA viruses may have been more promiscuous in the past. It remains to be seen what function these proteins serve in these *F. graminearum* strains. They could be involved in intra- or inter-species inhibition like KP4 or they may play a role in plant pathogenesis. If these KP4-like proteins are involved in fungal competition, then they could be a ready source of novel antifungal proteins for crop protection.

These novel proteins are akin to the bacterial colicins that lend the producing *Escherichia coli* strain a competitive advantage over the other members of the same species. The interesting wrinkle here is that the antifungal proteins are made by viruses and secreted by the host. Also in contrast to the colicins, these antifungal proteins are completely different to each other in amino acid sequence, structure, and modes of action. Further structural and functional studies may help identify the target proteins and those aspects of the antifungal proteins involved in specificity. In turn, this information may allow for the creation of antifungal proteins with a wider spectrum of activity and broader application in crops.

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